IN THE UNITED STATES PATENT AND TRADE MARK OFFICE

In re application of
Masaaki TERADA et al.
Application No.: 09/701,013 Art Unit: 1632
Filed: January 12, 2001 Examiner: Shin-Lin Chen
For: STABLE GENE PREPARATIONS

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Declaration Under 37 CFR § 1.132

Sir:

In connection with the above-identified U.S. patent application, I, Shunji Nagahara, a citizen of Japan and residing at 3-348, 2-1, Kuwatacho, Ibaraki-Shi, Osaka, 567-0841, Japan, say and declare as follows:

1. I received the degree of Ph. D. from the Department of Polymer Science and Engineering, Kyoto Institute of Technology in Japan in 1992.

2. I have worked at Department of Bioengineering, National Cardiovascular Center Research Institute from 1992 to 1994.

3. I have been working at Sumitomo Pharmaceuticals Research Center since 1994. I have been studying controlled release formulation and preparations.

4. I am a member of Controlled Release Society, The Pharmaceutical Society of Japan and The Japan Cancer Association.

5. I am an author or co-author of the papers of the list attached.

6. I am one of the inventors in U.S. Serial Number 09/701,013, and I am very familiar with the subject matter thereof and have been researching the subject matter thereof since 1994.

7. Materials
Cationic lipids, DOPE and DOTAP, were purchased from Avani Polar Lipids, Inc. pCMV-EGF containing a CMV promoter and green fluorescence protein gene (CLOTECH Laboratories) was employed as a plasmid DNA for evaluation of closed circular content. Organic acids and cryoprotectants were purchased from Nacalai Tesque. Atelocollagen was obtained from Koken Co., Ltd.

8. Preparation of test formulations and control formulations;
The claims of the present application stand rejected over WO96/40265 in the name of Szoka et al. taken together with Bonadio (USP 5,763,416) and Fujioka (USP 5,236,704). According to Szoka's description at page 6, line 16, test formulation-1 containing pCMV-EGF, DOTAP/DOPE, sodium citrate and atelocollagen was prepared as follows:

DOTAP/DOPE (1:1) liposome formulation containing 10mg/ml of cationic lipids was prepared by a rehydration method. 250ul of pCMV-EGF solution (1 mg/ml) containing 300mM NaCl and 20mM Tris-HCl(pH 7.4) was mixed with 250ul of DOTAP/DOPE (1:1) liposome formulation (10 mg/ml) followed by addition of 250ul of atelocollagen solution(1.2 mg/ml) and 250ul of sodium citrate solution (100 mg/ml). The final concentrations of the contents of the solution were pCMV-EGF, 250ug/ml; DOTAP/COPE, 2.5mg/ml; sodium citrate, 25mg/ml; atelocollagen, 307ug/ml. Test formulation 1 (TF-1) was obtained after lyophilization of the resulting mixture. Just after lyophilization and after keeping at 40 °C for two weeks, test formulation-1 was dissolved in water and treated with collagenase to degrade atelocollagen (which inhibits analysis by gel electrophoresis). After agarose gel electrophoresis of the obtained solution, the closed circular plasmid content of test formulation-1 was measured using a Fluor-S MultiImager (Bio Rad). Other test formulations and control formulations were prepared in same manner. Content of the test formulations and control formulations is summarized in Table 1.

Table 1: Content of test formulations and control formulations

formulation	additive	pCMV-EGF(ug)	DOTAP/DOPE(mg)	additive(mg)	atelocollagen(ug)
TF-1	sodium citrate	250	2.5	25	307
TF-2	sodium tartrate	250	2.5	25	307
CF-1	arginine	250	2.5	25	307
CF-2	glucose	250	2.5	25	307
CF-3	sucrose	250	2.5	25	307
CF-4	aspartic acid	250	2.5	25	307
CF-5	glutamic acid	250	2.5	25	307
CF-6	mannitol	250	2.5	25	307
CF-7	lactose	250	2.5	25	307
TF-3	sodium citrate	250	0	25	307
TF-4	sodium tartrate	250	0	25	307
CF-8	arginine	250	0	25	307
CF-9	glucose	250	0	25	307
CF-10	sucrose	250	0	25	307
CF-11	aspartic acid	250	0	25	307
CF-12	glutamic acid	250	0	25	307
CF-13	mannitol	250	0	25	307

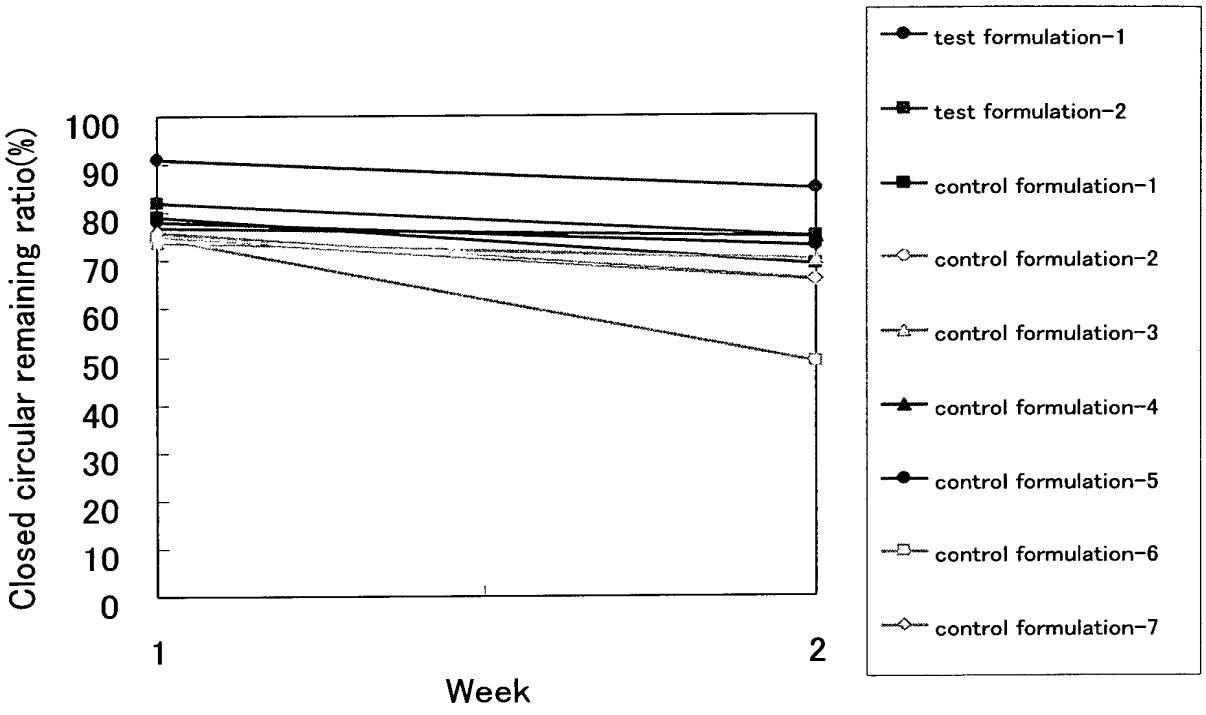
CF-14	lactose	250	0	25	307
TF-5	sodium citrate	12.3	0	3.1	307
TF-6	sodium tartrate	12.3	0	3.1	307
CF-15	arginine	12.3	0	3.1	307
CF-16	glucose	12.3	0	3.1	307
CF-17	sucrose	12.3	0	3.1	307
CF-18	aspartic acid	12.3	0	3.1	307
CF-19	glutamic acid	12.3	0	3.1	307
CF-20	mannitol	12.3	0	3.1	307
CF-21	lactose	12.3	0	3.1	307

TF, test formulation; CF, control formulation

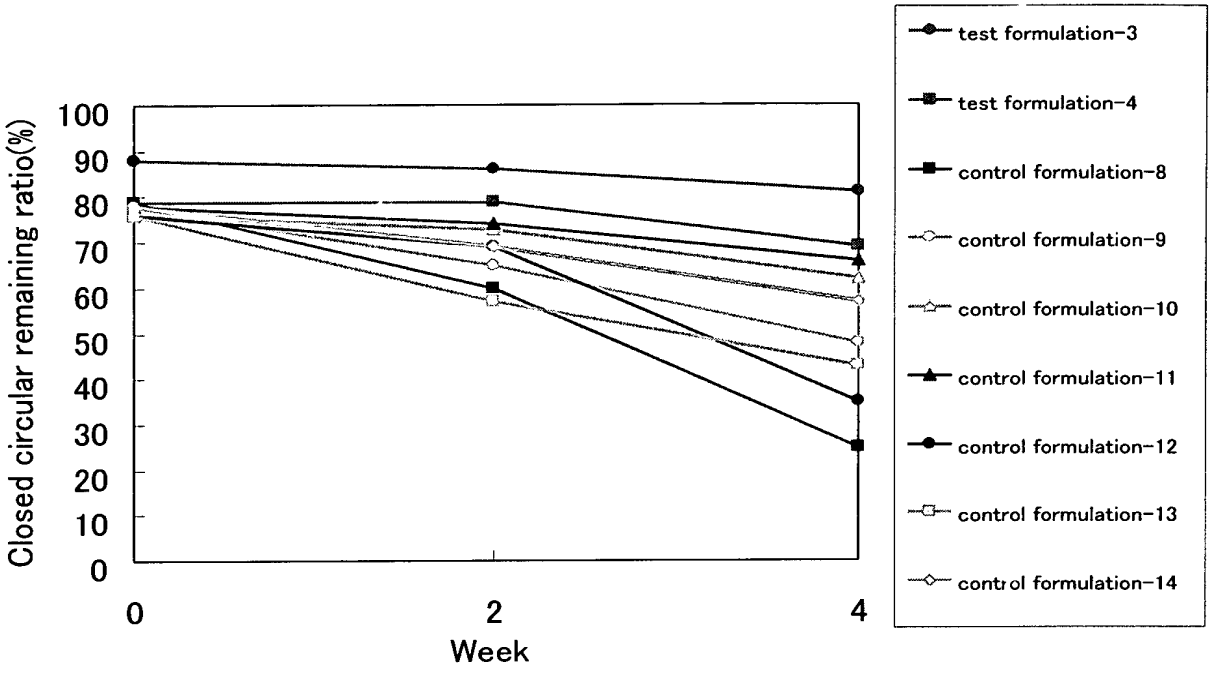
9.Results

In order to clarify that the stabilizing effect of organic acids such as citric acid and tartaric acid is superior to that of cryoprotectants described by Szoka et al. in WO96/40265 in the presence of atelocollagen, the stabilizing effect upon closed circular plasmid DNA was compared among organic acids and the cryoprotectants recited in Szoka. According to Szoka's description at page 3, line 31, lactose, sucrose, glucose, mannitol, arginine, aspartic acid and glutamic acid were used as cryoprotectants. In this study, three types of formulation containing atelocollagen, that is, lyophilized plasmid DNA associated with liposomes (test formulations 1 and 2, control formulation 1 to 7), high dose lyophilized naked plasmid DNA (test formulations 3 and 4, control formulations 8 to 14) and low dose lyophilized naked plasmid DNA (test formulations 5 and 6, control formulations 15 to 21) were prepared, and the proportion of plasmid DNA remaining in the closed circular form ("closed circular remaining ratio") that is contained in these formulations was measured just after their preparation and after keeping them at 40 °C for two weeks or four weeks. The proportion of plasmid DNA remaining in the closed circular form of test formulations and control formulations after lyophilization and storage is shown in Figure (a). Just after lyophilization, the proportion of plasmid DNA remaining closed circular of control formulation 1 to 7 is in the range of 74% to 79%, and the proportion decreases into the range of 49% to 75% after storage. On the other hand, the proportion of plasmid DNA of test formulation 1 remaining closed circular (91%) and of test formulation 2 remaining closed circular (82%) descreases to 85% and 75% respectively after storage. This result indicates that pDNA stabilizing effect of citric acid and tartaric acid is superior to the cryoprotectants claimed by Szoka. The same tendency was also observed between test formulations 3 and 4 and control formulations 8 through 14 (Figure (b)), and also between test formulations 5 and 6 and control formulations 15 through 21 (Figure (c)).

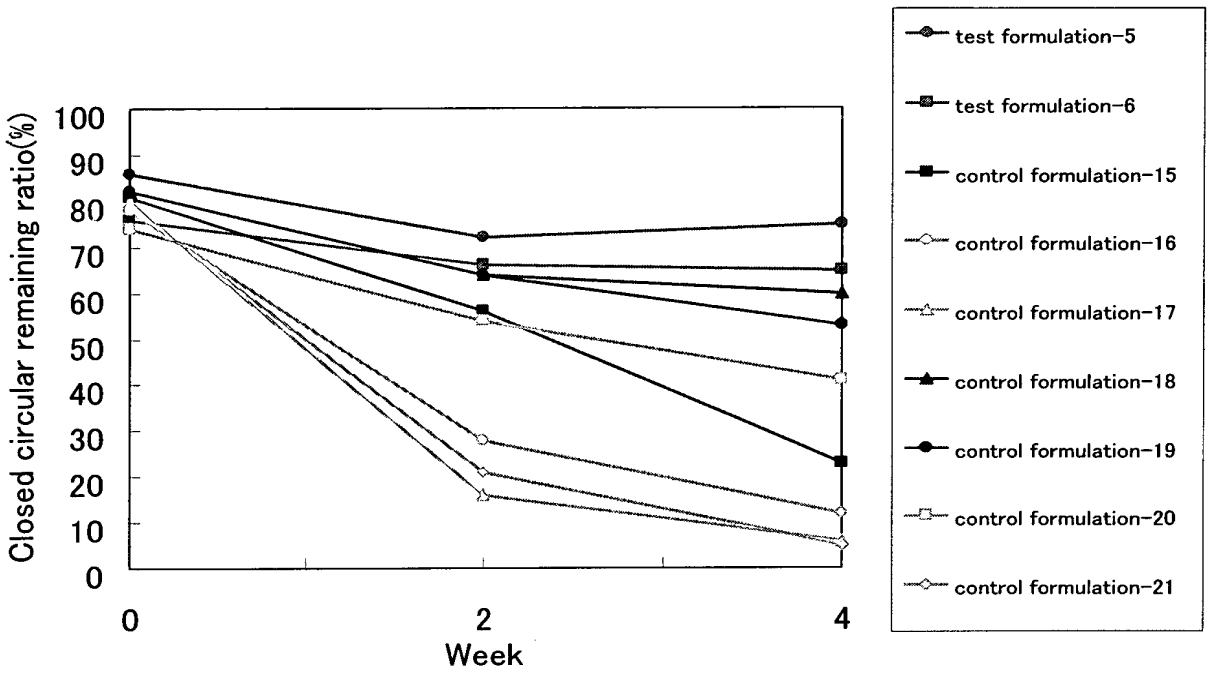
(a)


Closed circular remaining ratio(%)
100
90
80
70
60
50
40
30
20
10
0
1
2
Week
test formulation-1
test formulation-2
control formulation-1
control formulation-2
control formulation-3
control formulation-4
control formulation-5
control formulation-6
control formulation-7

(b)


Closed circular remaining ratio(%)
100
90
80
70
60
50
40
30
20
10
0
0
2
4
Week
test formulation-3
test formulation-4
control formulation-8
control formulation-9
control formulation-10
control formulation-11
control formulation-12
control formulation-13
control formulation-14

(c)



10. Conclusion

These results and Table 9 of the present specification show that the stabilizing effect of tartaric acid and citric acid during preparation and preservation of formulations of closed circular plasmid DNA are superior to the results obtained using the cryoprotectants described in Szoka. This result is one that is unexpected by one of ordinary skill in the art who would read Szoka et al. alone or in combination with Bonadio (USP 5,763,416) and Fujioka (USP 5,236,704).

11. The undersigned declares further that all statement made herein of his own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that Such willful false statement may jeopardize the validity of above identified application or any patent issuing thereon.

September 1, 2003

Shunji Nagahara

List of Publications

1) Atelocollagen-based gene transfer in cells allows high-throughput screening of gene functions, Honma K.; Ochiya T.; Nagahara S.; Sano A.; Yamamoto H.; Hirai K.; Aso Y.; Terada M., Biochem. Biophys. Res. Commun., 289, 5, 1075-1081, 2001

2) The application of biodegradable collagen minipellets as vaccine delivery vehicles in mice and sheep, Lofthouse S.; Nagahara S.; Sedgmen B.; Barcham G.; Brandon M.; Sano A., Vaccine, 19, 4318-4327, 2001

3) Collagen minipellet as a controlled release delivery system for tetanus and diphtheria toxoid, Higaki M.; Azechi Y.; Takase T.; Igarashi R., Nagahara S.; Sano A.; Fujioka K.; Nakagawa N.; Aizawa C.; Mizushima Y, Vaccine, 19, 3091-3096, 2001

4) Biomaterials for gene delivery: atelocollagen-mediated controlled release of molecular medicines, Ochiya, Takahiro; Nagahara, Shunji; Sano, Akihiko; Itoh, Hiroshi; Terada, Masaaki, Current Gene Therapy, 1, 31-52, 2001

5) Stable gene preparations, Terada, Masaaki; Ochiya, Takahiro; Sano, Akihiko; Hisada, Akihiko; Nagahara, Shunji, WO 9961063, 1999

6) New delivery system for plasmid DNA in vivo using atelocollagen as a carrier material: the Minipellet, Ochiya, Takahiro; Takahama, Yasushi; Nagahara, Shunji; Sumita, Yoshihiro; Hisada, Akihiko; Itoh, Hiroshi; Nagai, Yutaka; Terada, Masaaki, 5, 707-710, 1999

7) Immunopotentiating composition, Fujioka, Keiji; Sano, Akihiko; Nagahara, Shunji; Brandon, Malcolm Roy; Nash, Andrew Donald; Lofthouse, Shari, WO 9852605, 1998

8) Cell-substrate and cell-cell interactions differently regulate cytoskeletal and extracellular matrix protein gene expression, Nagahara, Shunji; Matsuda, Takehisa, J. Biomed. Mater. Res., 32, 677-686, 1996

9) Hydrogel formation via hybridization of oligodeoxyribonucleotides derivatized in water-soluble vinyl polymers, Nagahara, Shunji; Matsuda, Takehisa, Polym. Gels Networks, 4, 111-127, 1996

10) mRNA expression induced by cell-substrate interaction. A two-dimensional tissue formation process, Matsuda, Takehisa; Nagahara, Shunji, ASAIO J., 41, M398-M403, 1995

11) A total delivery system of genetically engineered drugs or cells for diseased vessels: concept, materials, and fabricated prototype device, Kito, Hiroyuki; Suzuki, Fumiaki; Nagahara, Shunji; Nakayama, Yasuhide; Tsutsui, Yoko; Tsutsui, Nobumasa; Nakajima, Nobuyuki; Matsuda, Takehisa, ASAIO J., 40, M260-M266, 1994

12) Oligonucleotides site-specifically spin-labeled at 5'-terminal or internucleotide linkage and their use in gene analyses, Murakami, Akira; Mukae, Masayuki; Nagahara, Shunji; Konishi,

Yuichiro; Ide, Hiroshi; Makino, Keisuke, Free Radical Res. Commun., 19, S117-S128, 1993

13) Detection of specific base sequences in solution using DNA probes labeled with D- and/or 15N-substituted spin-labels, Makino, Keisuke; Nagahara, Shunji; Konishi, Yuichiro; Mukae, Masayuki; Ide, Hiroshi; Murakami, Akira, Free Radical Res. Commun., 19, S109-S116, 1993

14) Fluorescent-labeled oligonucleotide probes: detection of hybrid formation in solution by fluorescence polarization spectroscopy, Murakami, Akira; Nakaura, Misuzu; Nakatsuji, Yuna; Nagahara, Shunji; Tran Cong Qui; Makino, Keisuke, Nucleic Acids Res., 19, 4097-4102, 1991

15) Characterization of antisense DNA derivatives having stereoisomeric linkages, Murakami, Akira; Uematsu, Hiroki; Tamura, Yutaka; Nagahara, Shunji; Ide, Hiroshi; Makino, Keisuke, Nucleic Acids Symp. Ser., 25, 133-134, 1991

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19) ESR behavior of spin-labeled oligoDNAs, Murakami, Akira; Nagahara, Shunji; Takeuchi, Tamio; Makino, Keisuke, Nucleic Acids Symp. Ser., 21, 89-90, 1989

20) A study on spin-labelled oligonucleotide synthesis and its electron spin resonance behavior in solution, Makino, Keisuke; Murakami, Akira; Nagahara, Shunji; Nakatsuji, Yuna; Takeuchi, Tamio, Free Radical Res. Commun., 6, 311-316, 1989

21) Preparation of spin-labeled oligonucleotides and their spectroscopic behavior in solution, Makino, Keisuke; Murakami, Akira; Nagahara, Shunji; Nakatsuji, Yuna; Takeuchi, Tamio, Nucleic Acids Symp. Ser., 20, 89-90, 1988

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